SERUM DEPRIVATION ALTERS LIPID PROFILE IN HN9.10e EMBRYONIC HIPPOCAMPAL CELLS

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ABSTRACT

The understanding of the mechanism of apoptosis is important to improve the use of stem cells for the treatment of neurodegenerative disorders. Sphingolipids are bioactive molecules involved in the regulation of cell fate. In HN9.10e embryonic hippocampal cells, serum deprivation induces apoptosis preceded by sphingomyelinase activation and raise of ceramide levels. Increasing evidence indicates that individual ceramide species regulated by specific pathways in distinct subcellular compartments might carry out distinct cellular functions but the ceramides species involved in embryonic hippocampal cell death induced by growth factor deprivation are unknown. In the present paper, by using the UFLC-MS/MS methodology, we have investigated the effect of serum deprivation on the lipid profile in HN9.10e cells. At 48 h of serum deprivation we detected a decrease in cholesterol and increase in sphingosine-1-phoshate 18:1, phosphatidylcholine 18:1 18:0, sphingomyelin 18:1 16:0 and in ceramides 18:1 16:0; we also found an increase in saturated/unsaturated fatty acid ratio in sphingomyelin. We hypothesize that the rearrangement of sphingo- and glycerolipids with increase of saturated fatty acids in serum-deprivated neural cells might represent a cellular response aimed at holding cholesterol inside the cells.

Highlights

Serum deprivation increases Ceramide 16:0 in embryonic hippocampal cells

Serum deprivation decreases cholesterol in embryonic hippocampal cells

Serum deprivation increases sphingosine-1- phosphate in embryonic hippocampal cells

Keywords

ceramide species, serum deprivation, cholesterol, sphingosine-1-phosphate, embryonic hippocampal cells, sphingomyelin

Abbreviations: Cer, ceramide; CHO, cholesterol; DAG , diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide; PC, phosphatidylcholine; PPC, phosphorylcholina; SM, sphingomyelin; Sph, sphingosine; Sph1P, sphingosine-1-phosphate.

1. Introduction

Cell transplantation has been shown to be an effective therapy for central nervous system disorders in animal models. However, certain challenges remain to be resolved before the use of stem cells for neurological diseases can be fully realized, which include the need to optimize survival and function of neural derivatives upon long-term differentiation *in vitro* and *in vivo* [1]. As apoptosis plays a central role in the fate of transplanted cells, it is necessary to increase the knowledge of the mechanisms of apoptosis of stem cells and neural progenitors.

Sphingolipids are structural and bioactive molecules involved in the regulation of proliferation, survival, differentiation and cell death [2]. Sphingolipids include several classes of molecules, such as sphingosine (Sph), sphingosine-1-phosphate (Sph1P), ceramide (Cer), glucosylceramide, and sphingomyelin (SM) [3]. In particular sphingomyelin (SM) influences cell fate by regulating growth, survival, differentiation and apoptosis [4]. SM is synthesized by sphingomyelin-synthase (SM-synthase) by using phosphatidylcholine (PC) as source of phophorylcholine (PPC) and releasing diacylglycerol (DAG); SM is catabolized by sphingomyelinase (SMase) producing ceramide and PPC. In addition, SM can be used as source of PPC for PC synthesis, in the presence of DAG, releasing ceramide by reverse-sphingomyelin-synthase [5]. It is worthy to note that the crosstalk between glycerophospholipids and sphingolipids controls important lipid mediators such as ceramide and DAG [6]; sphingolipid and PC are interconvertible in biological systems and their relative levels are important in determining cell fate [4].

Furthermore, hydrolysis of PC by phospholipase D is a source of choline, which is not only used for the synthesis of the neurotransmitter acetylcholine in cholinergic neurons [7], but it is possibly involved in maturation and differentiation of astroglial cells *in vitro* independently of acetylcholine [8,9]. The multiple pathways of ceramide generation led to the hypothesis that individual ceramide molecular species are regulated by specific biochemical pathways in distinct subcellular compartments and they carry out distinct functions [10]. Saturated fatty acids of SM favour the formation of Van der Waals interactions between SM and cholesterol (CHO) [5,11] to form membrane lipid rafts that act as platforms for specific protein and lipid sorting and regulators of cell signalling.

We have previously demonstrated that serum deprivation induces a time-dependent decrease in cell viability and increase in the cell percentage in G1 phase of the cell cycle via SM metabolism [12-15]. Serum deprivation is able to enhance spontaneous or growth factor-induced differentiation in neural cells, including astrogial cells [16,17] but no data exist on the effect of serum deprivation on lipid profile so far. In the present paper we have investigated this aspect in HN9.10e cells by UFLC-MS/MS. We have found a strong decrease of CHO and an increase of saturated PC, ceramide and Sph1P; we also observed an increase in SM saturated/unsaturated fatty acid ratio. We hypothesize that the low level of intracellular CHO might result from the failure of incorporation into the cells due to lack of its availability during serum deprivation. We hypothesize that the rearrangement of sphingo- and glycerolipids and the increase of saturated fatty acids in response to the loss of CHO might represent a response to hold CHO inside the cell.

2. Material and Methods

2.1. *Reagents*

Dulbecco's modified Eagle's medium (DMEM), methanol, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2-propanol, metyl-tert-butyl ether, formic acid, chloroform, cholesterol (CHO) were obtained from Sigma Chemical Co. (St. Louis, MO, USA.); SM 18:1 12:0, SM 18:1 16:0, SM 18:1 18:1, SM 24:0, ceramide 18:1 16:0, ceramide 18:1 18:0, ceramide 18:1 20:0, ceramide 18:1 24:0 were purchased from Avanti (Avanti Polar, Alabaster, AL, USA). Fetal calf serum was from Euroclone (Pero, Milano, Italy).

2.2. Cell culture

HN9.10e cells were were grown in DMEM, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B (fungizone). Cells were maintained at 37 °C in a saturating humidity atmosphere containing 95% air 5% CO_2. Cells were plated in 10 cm ∅ dishes and incubated with 10% (control) or 0.2% fetal calf serum for 48 h (deprived) as previously described [12-14] and frozen immediately.

2.3. Lipid extraction and separation

Lipids were extracted as in [18]. Lipid species were separated and measured by ultraperformance liquid chromatography tandem mass spectrometry [19] by using Ultra Performance Liquid Chromatography system (LC20AD prominence, SIL20AC ht

autosampler, DGU20A_3 degasser, CTO20A column oven) tandem Mass Spectrometer Applied Biosystem (Shimadzu Italy s.r.l., Italy). The samples were separated on a Phenomenex Kinetex phenyl-hexyl 100 A column (50 x 4,60 mm diameter, 2.6 μm particle diameter) with precolumn security guard Phenomenex ULTRA phenyl-hexyl 4.6. For sphingomyelins, column temperature was set at 50°C, flow rate 0.9 mL/min. Solvent A was 1% formic acid; solvent B 100% isopropanol containing 0.1% formic acid. Run was performed for 3 min in 50% solvent B and then gradient to reach 100% B in 5 min. The system has been reconditioned for 5 min with 50% B before the next injection.

The sphingomyelin species were identified by using positive turbo ion spray and modality multipole reaction monitoring. The identification and analysis of CHO was conducted by atmosferic pressure chemical ionization (APCI) in positive ionization conditions and multipole ion scan modality as described in [20].

2.4. Cell viability

Ten thousand cells were plated in 96-well dishes, and cultured in the presence or absence of Sph1P in medium containing 0.2 % , 1% and 10% FBS for 48 h. Cell viability was measured as MTT reduction as previously described [12-13]. Briefly, 0.5 mg/ml final concentration MTT was added to the culture medium, plates were incubated at 37°C for 30 min and then medium was replaced with 100 μl dimethylsulfoxide to solve the formazan salts. Absorbance was measured at 570 nm and values were normalized considering the average of absorbance of the control sample.

2.5. Protein content

Total protein concentration was determined spectrophotometrically at 750 nm by using bovine serum albumin as a standard according to [21]. The method combines the reactions of copper ions with the peptide bonds under alkaline conditions and the oxidation of aromatic aminoacids by the Folin–Ciocalteu reagent.

2.6. Statistical analysis

Data are expressed as mean ± S.D. and *t* test was used for statistical analysis .

3. Results

3.1. Serum deprivation induces changes in the content of cholesterol, sphingomyelin, ceramide and phosphatidylcholine

We have analyzed for the first time the change of lipid profile in HN9.10e cells after serum deprivation by using UFLC-MS/MS systems, a methodology that allows to obtain simultaneous quantitative analysis of CHO, PC and sphingolipids [20]. Serum deprivation induces a decrease in CHO content to 48% of control samples (Fig.1a). We have also analyzed the most common PC, ceramide and SM species by using external calibrators for each species (PC 16:0 18:1, PC 16:0 20:4, PC 18:1 18:0, ceramide 16:0, ceramide 18:0, ceramide 20:0, ceramide 24:0, SM 16:0, SM 18:1, SM 24:0) (Fig. 1 b-d). We have observed that serum deprivation induces an increase in the three species of PC analyzed, in SM 16:0 and 24:0, in ceramide 16:0 and a decrease in ceramide 24:0. No

significant variation of SM 18:1 and ceramide 20:0 has been found. Serum deprivation increases Sph1P content 1.89-fold compared to control (Fig. 1f). Exogenously added 0.1 μM Sph1P does not modify the viability of cells grown on 10% containing medium or serum-deprived cells, as measured by MTT reduction assay However, 0.1 μM Sph1P does reduce viability when cells are cultured in 1% FBS for 48 h. The viability of cells cultured in low serum was 0.33 ± 0.13 compared to cells cultured in 10% FBS for the same period (1.00 ± 0.22), whereas the presence of Sph1P reduced viability to 0.22 ± 0.09 (n=5 independent experiments performed in octuplicate, $p<0.05$).

3.2. Serum deprivation-induced changes in SM species profile.

To have a deeper insight of SM species containing saturated or unsaturated fatty acids, we have evaluated the areas of all the peaks identified on the basis of their molecular weight. A total of 55 species were investigated: SM 2:0, SM 4:0, SM 6:0, SM 8:0, SM 10:0, SM 10:1, SM 10:2, SM 10:3, SM 12:0, SM 12:1, SM 12:2, SM 12:3, SM 14:1, SM 14:2, SM 14:3, SM 16:2, SM 18:2, SM 18:3, SM 20:1, SM 20:2, SM 20:3, SM 22:3, SM 22:4, SM 22:5, SM 24:5, SM 24:6, SM 26:1, SM 26:5, SM 26:6, SM 28:0- SM 30:8 were absent. Twenty-four peaks were detected. Significant differences in the levels of various lipid molecular species were found between control and serum-deprived samples (Table 1). We have compared the changes in the levels of SM species containing saturated and unsaturated fatty acids. As reported in Fig. 2 the SM saturated fatty acids were 1.24-fold higher and SM unsaturated fatty acid 1.46 lower in serum-deprived samples than in controls. Thus, saturated /unsaturated fatty acid ratio was 2.02 in controls and 3.68 in serum-deprived samples (Fig. 2a). Among unsaturated

fatty acids, the monounsaturated, two-unsaturated and four-unsaturated decreased 2.13-fold, 2.71-fold, and 1.71-fold respectively whereas three-, five- and six- unsaturated did not change significantly (Fig. 2b).

4. Discussion

Serum deprivation decreases significantly CHO content in HN9.10e cells. This reduction might be due to the lack of availability of CHO in the culture medium. It has been demonstrated that cultured cells use CHO present in the medium to proliferate, and that high CHO levels stimulate cell growth whereas CHO synthesis inhibitors arrest cell cycle progression and alter differentiation and/or apoptosis [22]. It is known that CHO is linked by Van der Waals interaction to SM and, more weakly, to PC to form lipid rafts that interact with a variety of proteins involved in important cell functions [4]. CHO plays a crucial role in the generation of rafts, ordered domains in the plasma membrane that laterally segregate certain proteins, thus reducing their rate of lateral diffusion, allowing clustering and, consequently, signaling strength. Decrease in CHO may modify signal transduction mediated by raft-localized receptors. Under low CHO conditions, mature hippocampal neurons exhibit increased TrkB activity, the most prominent neurotrophin receptor expressed in hippocampus [23]. CHO loss seems to induce neuronal survival in both acute and chronic stress [24]. Whether this protective mechanism is also working in embryonic hippocampal neurons is not known.

Since SM metabolism plays a critical role in serum deprivation-induced apoptosis in HN9.10e cells, we have analyzed the relative levels of the various sphingolipids and PC. In particular, serum withdrawal increases ceramide 16:0 content

2.26-fold, decreases that of ceramide 24:0 without modifying ceramide 20:0 content compared to control cells. A raise in ceramide 16:0 has been observed in a variety of apoptosis-induced models including radiation-induced apoptosis and hormone deprivation in prostate cancer cells [25-26]. In these models of apoptosis, a later increase in ceramide 24:0 has been found [27]. By contrast, this species decreases (0.15-fold that of control cells) after 48 h of serum deprivation while SM 24:0 increases.

Our data clearly show an increase in SM containing saturated long-chain fatty acids (22:0, 24:0, 26:0) (Table 1) and a decrease in SM containing unsaturated fatty acids. It is worth noting that we have not found any significant change in SM-synthase activity 48 hours after serum deprivation when we have used a pool of ceramides as substrate [14]. Possibly, this result could reflect, at least in part, the distinct effects of serum deprivation on different SM species. Whether this result is due to a failure of putative serum-deprived activated SM-syntase(s) to use this mixture as substrate is not clear for the moment. Differently, the reduction of almost all species of unsaturated SM might be due to the high activity of SMase found following 48 hours of serum deprivation [14]. Since saturated SM binds CHO to form the lipid rafts and the depletion of CHO disorganizes these structures [28], it is possible to hypothesize that the reduction of CHO incorporation in HN9.10e cells, due to lack of availability in serum-deprived culture medium, induces an increase of saturated long-chain SM to capture the intracellular CHO. This could be a response of the cell to ensure the presence of lipid rafts in the membranes, important for signal transduction, in order to limit the serum deprivation-induced damage.

Serum deprivation increases Sph1P content 1.89 -fold compared to controls. Sph1P has been usually involved in proliferation and survival. However, addition of

Sph1P reduces viability of HN9.10e cells (this paper) and primary hippocampal cells [29]. Anti-apoptotic effects of Sph1P appear to be correlated with the action of sphingosine kinase 1. It has been reported that Sph1P induces neuronal death when generated by sphingosine-kinase2 and when Sph1P is accumulated due to Sph1P-lyase deficiency [30].

In summary, we have identified some modifications of the lipid profile in serum-deprived HN9.10e cells. The decrease in CHO suggests that reorganization of rafts might play an important role. Increasing knowledge on the lipid species involved in serum deprivation-induced apoptosis might be useful for improving neuroblast survival after stem cell therapy in neurodegenerative diseases.

Acknowledgments

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Disclosure Statement

Authors declare no conflicts of interest.

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Legends

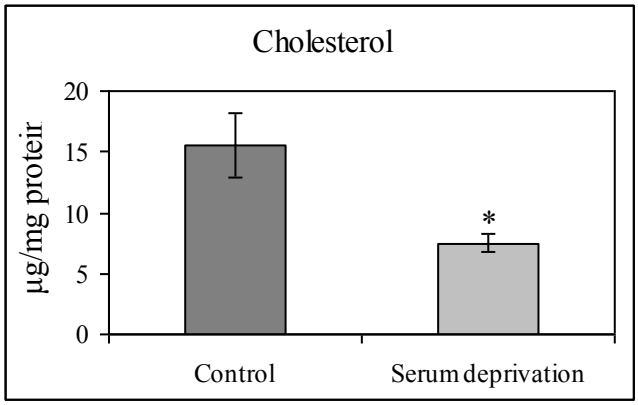
Fig. 1. Effect of serum deprivation on different lipid species.

Cells were serum-deprived for 48 or grown in 10% FBS. Lipids were quantified as described in Materials and Methods. Data are expressed as mean ± SD (n=5)

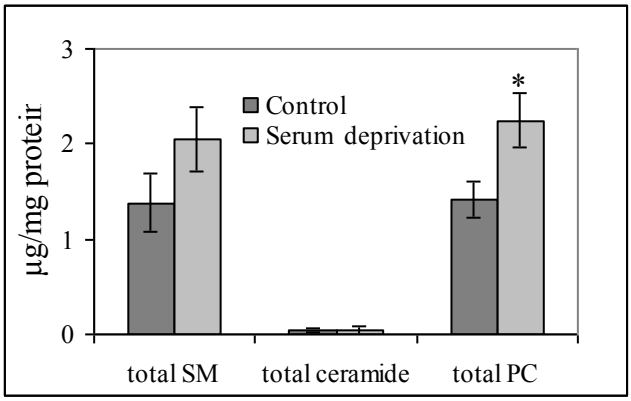
Fig. 2. Quantification of fatty acids of sphingomyelin

Cells were serum-deprived for 48 or grown in 10% FBS. Lipids were quantified as described in Materials and Methods. Data are expressed as as mean ± SD (n=5) . Sat: saturated; Unsat: unsaturated.

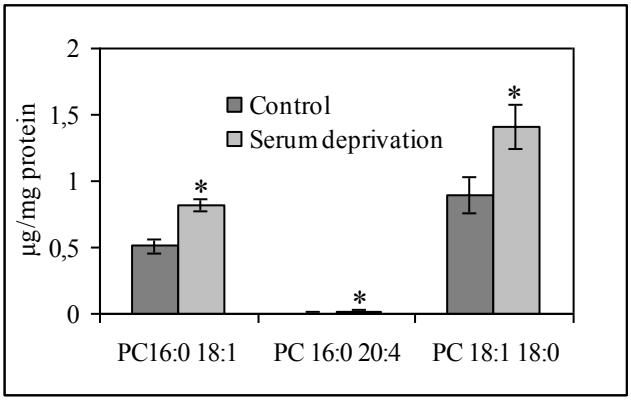
Fig. 1


Cholesterol
µg/mg proteir
20
15
10
5
0
*
Control
Serum deprivation

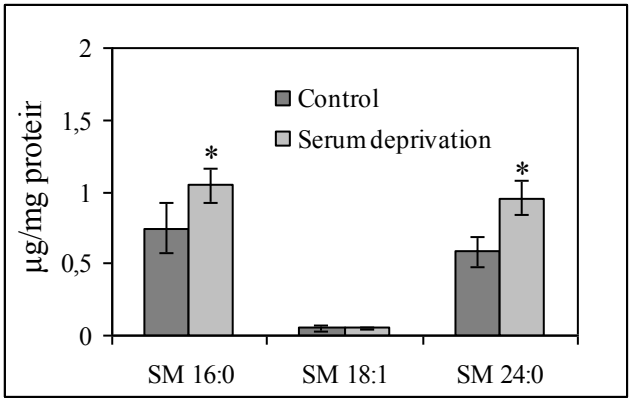
a)


µg/mg proteir
3
2
1
0
Control
Serum deprivation
*
total SM
total ceramide
total PC

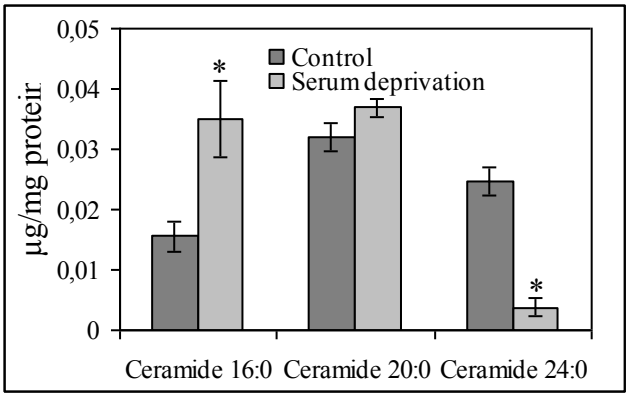
b)


µg/mg protein
2
1,5
1
0,5
0
Control
Serum deprivation
*
*
*
PC16:0 18:1
PC 16:0 20:4
PC 18:1 18:0

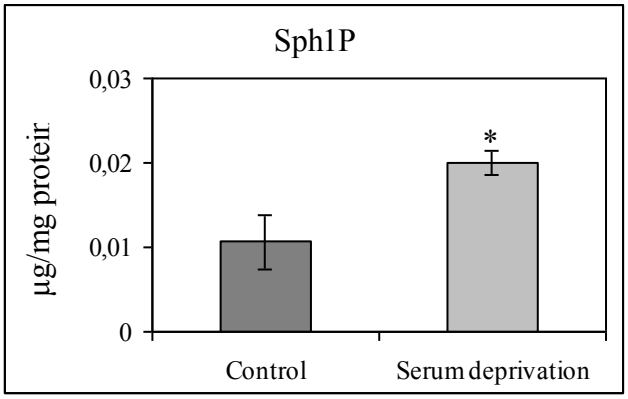
c)


µg/mg proteir
2
1,5
1
0,5
0
Control
Serum deprivation
*
*
SM 16:0
SM 18:1
SM 24:0

d)

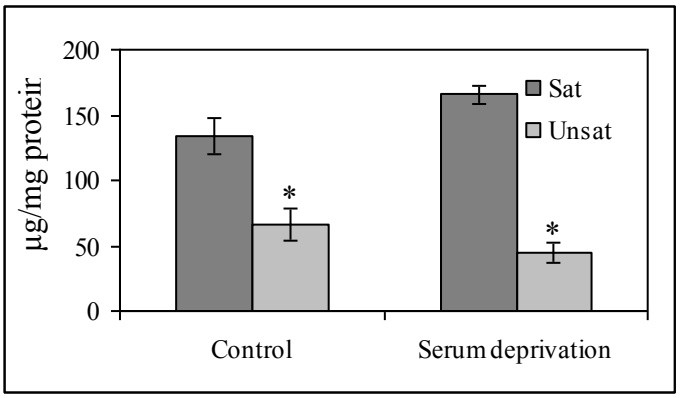

µg/mg proteir
0,05
0,04
0,03
0,02
0,01
0
Control
Serum deprivation
*
*
Ceramide 16:0
Ceramide 20:0
Ceramide 24:0

e)

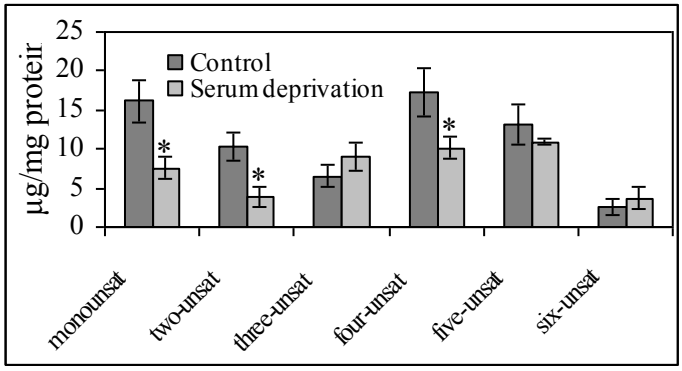

Sph1P
µg/mg proteir
0,03
0,02
0,01
0
*
Control
Serum deprivation

f)

Fig. 2


200
150
100
50
0
µg/mg proteir
Sat
Unsat
*
*
Control
Serum deprivation

a)


25
20
15
10
5
0
µg/mg proteir
Control
Serum deprivation
*
*
*
monounsat
two-unsat
three-unsat
four-unsat
five-unsat
six-unsat

b)

Table 1
Composition of SM

SM species	control	Serum deprivation	Significance
Saturated			
SM 14:0	1.60 ± 0.45	0.79 ± 0.66	ns
SM 16:0	12.68 ± 1.65	17.25 ± 0.75	p< 0.05
SM 18:0	7.18 ± 2.68	9.45 ± 1.02	ns
SM 20:0	34.12 ± 4.63	26.85 ± 1.03	p< 0.05
SM 22:0	56.47 ± 4.01	76.67 ± 2.22	p< 0.001
SM 24:0	17.88 ± 0.27	26.80 ± 1.68	p< 0.001
SM 26:0	3.52 ± 0.10	7.85 ± 0.53	p< 0.001
Unsaturated			
SM 16:1	1.56 ± 0.86	1.06 ± 0.15	ns
SM 16:3	0.73 ± 0.05	4.38 ± 0.65	p< 0.001
SM 16:4	2.77 ± 0.36	3.10 ± 0.16	ns
SM 18:1	1.03 ± 0.20	0.86 ± 0.17	ns
SM 18:4	3.74 ± 0.95	1.79 ± 0.25	p< 0.01
SM 20:4	4.18 ± 0.61	2.88 ± 0.46	ns
SM 20:5	13.40 ± 2.57	10.90 ± 0.32	ns
SM 22:1	6.71 ± 0.24	1.96 ± 0.72	p< 0.001
SM 22:2	3.24 ± 0.63	1.58 ± 0.99	p< 0.05
SM 22:6	2.59 ± 0.92	3.69 ± 1.44	ns
SM 24:1	6.82 ± 1.52	3.68 ± 0.39	p< 0.01
SM 24:2	5.11 ± 0.95	1.50 ± 0.16	p< 0.001
SM 24:3	3.46 ± 1.12	2.79 ± 0.87	ns
SM 24:4	3.93 ± 0.82	1.02 ± 0.20	p< 0.001
SM 26:2	1.94 ± 0.28	0.71 ± 0.16	p< 0.05
SM 26:4	2.72 ± 0.38	1.33 ± 0.28	p< 0.01

Legend Table 1.

SM were quantified as described in Materials and Methods. Data are expressed as mean ± SD (n=5) control or serum deprived samples (μg/mg protein).